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Birmingham, Alabama 35294-0111

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Patricia A. Anderson 8/4/02

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**13. ABSTRACT** *(Maximum 200 words)*

The identification and characterization of novel breast tumor antigens may be important for detection and diagnostics, as well as in development of effective immunotherapy strategies. Because cancer patients exhibit both humoral and cellular immunity to tumor associated proteins, patient sera provides a valuable reagent for the identification of potentially immunogenic tumor associated antigens. We have used serological analysis of recombinant cDNA expression libraries (SEREX) for the identification of tumor antigens in a mouse model of adenocarcinoma and in human breast cancer. Studies in a mouse model identified two tumor antigens which were characterized at the molecular level and evaluated for their ability to elicit an immune response. To extend these studies to human breast cancer, cDNA expression libraries were derived from patient tumor samples and screened with autologous and allogeneic sera. A reactive clone was identified and has been characterized. To date, characterization has included analysis of DNA sequence, RNA expression in normal and tumor tissue, and studies of reactivity with sera from allogeneic cancer patients. The longterm goal of this work is to develop this novel antigen as a potential diagnostic and prognostic marker, and to evaluate its potential as a target for immunotherapy approaches to breast cancer.

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Theresa Hutton for Tracy Hampton — 8/28/99

PI - Signature — Date

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## INTRODUCTION

The identification and characterization of novel breast tumor associated antigens (TAAs) has potential application to several aspects of breast cancer research. Such antigens may be useful for the detection and diagnosis of breast cancer, as expression may signal the transition from normal to tumorigenic cells. In addition, characterization of tumor associated antigens may contribute to our understanding of the interaction of cancer and the immune system, including the mechanisms by which tumor cells escape immune surveillance. Cancer immunotherapy approaches are likely to be improved with better characterization of the spectrum of proteins capable of inducing immune responses. Finally, tumor associated antigens isolated in this way may be rapidly translated into polynucleotide immunization strategies for the treatment of breast cancer. Our research is focused on identifying novel breast tumor associated antigens using a serological approach to antigen detection. It has been shown that tumor bearing individuals often develop a limited immune response to their tumor. The production of antibodies directed against tumor antigens has been described for both mutant antigens, such as p53 (1, 2) as well as for nonmutant antigens such as erbB-2 (3). We proposed to use patient sera as a reagent to identify breast tumor antigens. This approach has been termed SEREX (4-6), for serological identification by recombinant expression cloning. Our specific aims are as follows:

1. Identify TAAs on breast cancer cells by screening with sera from autologous allogeneic patients
2. Characterize the discovered TAAs
3. Evaluate the utility of these TAAs in the context of polynucleotide vaccination by assessing immune responses following injection of DNA encoding these TAAs.

We first used this strategy in a mouse model of adenocarcinoma to validate the approach in our hands. Two tumor antigens were identified in the murine adenocarcinoma line, MC38. Because our longterm goal is to use the human breast tumor antigens we identify in a vaccine strategy for immunotherapy, we have also investigated the ability of antigens identified through SEREX to act as tumor rejection antigens in a mouse model. Vaccine studies using polynucleotide immunization of these antigens indicates that intramuscular delivery of plasmid DNAs encoding these self antigens is capable of breaking tolerance but, to date, protective antitumor immunity has not been observed. We extended SEREX analysis to human breast cancer and have identified a candidate tumor antigen using a patient sample with autologous sera. This antigen has been partially characterized at the molecular level. The remaining work will focus on completing molecular analysis and evaluating the immunogenicity of this antigen.

**Statement of Work:**

Aim #1:
Months 1-12

1. Obtain tissue and sera from UAB's tissue procurement facility. Create cDNA library from breast cancer cells and screen with patient sera **(completed)**

Aim #2:
Months 6-24

1. Plaque purify positive clones, and sequence cDNA insert **(completed)**
2. Analyze sequence and compare to databases **(completed)**
3. Perform Northern blot analysis to study RNA expression pattern in normal and malignant tissue **(completed)**

Aim#3
Months 18-36

1. Transfect carcinoma cell clones with phagemids encoding the TAA cDNA fragments. Immunize mice with these phagemids and subsequently challenge with the stably transfected carcinoma cell clones in order to test for antitumor effects of PNV. **(initiated in modified form)**

## BODY

**A. Characterization of tumor antigens in a mouse model of adenocarcinoma.** We have completed studies of tumor antigen identification in a mouse adenocarcinoma cell line (MC38), which we used as a model system to validate the SEREX approach (see appendix: Hampton et al, Cancer Gene Therapy, in press). Using SEREX, we identified the two tumor antigens in this mouse model: the envelope protein of the murine endogenous leukemia virus (env) and the ATRX protein. By Northern blot analysis, expression of the envelope sequence was not detected in normal colonic epithelium, but it is expressed in a variety of murine tumor cell lines, including both spontaneously arising and carcinogen-induced tumors (**Hampton et al, figure 3**). Expression is seen in tumors arising from different tissues, including those derived from mammary carcinoma (4T1 and 4T07), and from different strains of mice. These features suggest the env protein may act as a shared tumor antigen. Southern blot analysis revealed the presence of novel bands in the MC38 DNA compared to genomic DNA from a C57BL/6 mouse suggesting that the *env* sequences have undergone amplification and reintegration into the MC38 genome (**Hampton et al., figure 4**). Sequence analysis of the full length envelope sequence revealed no mutations (7), indicating that this is a nonmutated tumor antigen, presumably immunogenic based on its expression in the tumor cells. The ATRX protein is a nuclear protein thought to be involved in transcription and DNA repair. In humans, this zinc finger helicase is known to regulate expression of several target genes, including α globin, perhaps by an effect on chromatin conformation (8). It has not previously been described as a tumor antigen. As expected, the *ATRX* transcript was found to be expressed in normal epithelium, and it is not overexpressed in MC38 cells (**Hampton et al, figure 3**), although it is highly expressed in the B16 melanoma cell line. It was found to expressed at low levels in the mammary cancer cell line 4T1 (**Hampton et al., figure 3**). Southern blot analysis revealed no structural rearrangements in the genomic ATRX in MC38 cells or in B16 cells (**Appended Figure 1**). Sequence analysis of the portion of the ATRX gene which we isolated (approximately 2.8 kb) revealed no mutations. Thus, ATRX may be immunogenic based on mutations outside of this region, mislocalization of the protein, or based on homology with another tumor antigen. We used sera from ATRX-immunized mice in immunofluorescent studies, but were not able to demonstrate mislocalization of the protein (data not shown). Additional studies will be needed to determine the basis of ATRX immunogenicity in this model, as well as to determine if human ATRX acts as a tumor antigen.

Because the longterm goal of this work is to develop human breast tumor antigens as vaccines for immunotherapy, we examined the ability of polynucleotide immunization against these putative tumor antigens to break tolerance and induce an antitumor immune response in a mouse model. Mice were immunized with a cDNA clone encoding the envelope sequence and the cloned portion of the ATRX gene. The DNA constructs were first evaluated in *in vitro* transcription/translation experiments to validate production of the encoded antigen (**Appended Figure 2**). Immunization against these self antigens resulted in strong production of anti-envelope and anti-ATRX antibodies (**Hampton et al., figure 5**). Isotype analysis of the antibody response (below) demonstrated induction of several different IgG isotypes, suggesting both T-helper-1 and T-helper-2 like responses were elicited:

Table 1. Isotype analysis of sera from four mice immunized with envelope-encoding plasmid DNA.

| Isotype | mouse 1 | mouse 2 | mouse 3 | mouse 4 |
|---|---|---|---|---|
| IgG1 | +++ | ++ | +++ | + |
| IgG2a | ++ | ++ | ++ | ++ |
| IgG2b | ++++ | ++++ | +++ | +++ |
| IgG3 | - | - | - | - |

Despite a strong humoral immune response, the immune response to these antigens was not sufficient to protect against tumor challenge (**Appended Figure 3,** and three additional immunization experiments,

not shown). To demonstrate that this lack of efficacy was not due to loss of antigen expression, Northern blot analysis was performed on tumors excised from immunized and challenged animals. Despite their rapid growth in immunized animals, tumors did not lose expression of the candidate TAAs (**Appended Figures 4 and 5**). These studies suggest that polynucleotide immunization alone may not be sufficiently effective to elicit antitumor immune responses. In ongoing work in our laboratory and in collaboration with members of the immunotherapy group at UAB's Cancer Center (and outside the scope of this grant), we are exploring novel vectors for dendritic cell transduction. We believe this work will be important in developing a effective strategy generating antitumor immunity in human breast cancer patients and will directly benefit the studies supported by this grant. Our goal is to develop optimized vectors for immunization which may be useful eventually for immunization of patients with the breast tumor antigen described below. Also of note, the finding that endogenous retroviral proteins act as tumor antigens has led us to hypothesize that human endogenous retroviral proteins may act as tumor antigens in breast cancer. Studies have been initiated in collaboration with Dr. Feng Wang-Johanning at our institution, who has recently received funding to study endogenous retroviral proteins as tumor antigens based.

**B. Identification and characterization of a human breast tumor associated antigen.** Having validated SEREX as a comparatively rapid means of identifying potential tumor antigens in a mouse model, these studies were extended to human breast cancer. Three breast cancer libraries were derived from patient tumor tissue and screened with autologous and allogeneic sera. Screening of greater that $10^6$ plaques from each of the breast tumor-derived cDNA libraries resulted in the detection of one positive plaque reactive with autologous sera (S3.2) which was chosen for further study. Isotype analysis revealed a predominantly IgG1 isotype, suggesting a mature immune response. Molecular characterization has been performed as below:

Sequence and Homology
The S3.2 insert was sequenced in its entirety and found to have homology with several described ESTs (**Appended Figure 6**). As described below, several short open reading frames were identified, however, the relevant open reading frame and epitope have not yet been determined.

Characterization of antibody response:
Immunoscreening of clone S3.2 against breast tumor patient sera, patient sera from individuals with other tumor types and normal human sera demonstrated the following:

Table 2. Analysis of reactivity to S3.2 in breast cancer patients, cancer patients and normal human sera.

| Serum Sample | Number Positives / Number Tested |
|---|---|
| Breast Cancer Patients | 2 / 30 |
| Ovarian Cancer Patients | 0 / 3 |
| Colon Cancer Patients | 0 / 7 |
| Melanoma Patients | 0 / 3 |
| Head and Neck Ca. Patients | 0 / 7 |
| Normal Human Sera | 0 / 30 |

This suggests that this antigen may be recognized at a low frequency in breast cancer patients, whereas reactivity to this antigen has not been demonstrated in patients with other types of cancer, nor in normal, healthy individuals.

Expression Studies:
Northern blot analysis revealed that this insert (or a portion of this insert ) was expressed in normal breast tissue as well as primary breast tumor tissue (not shown, previously reported). Several attempts have been made thus far to determine the immunogenic polypeptide. Although the cDNA library vector primarily produces fusion proteins, sequence analysis demonstrated that the insert of this

clone was not in frame with the vector fusion protein. Presumably, a internal start site is utilized in the bacterial system. We performed *in vitro* transcription translation reactions (TnT system, Promega) and western blots or immunoprecipitation with patient sera in an attempt to determine the size of the encoded, immunogenic polypeptide, but have not been successful to date. We will concentrate on cloning the open reading frames found in this insert into an expression vector (the same Stratagene lambda Zap vector which permits both prokaryotic and eukaryotic expression) to facilitate these studies.

**C. Immune response analysis.** To better evaluate the immunogenicity of candidate tumor antigens, we have developed, in collaboration with other members of UAB's Comprehensive Cancer Center, ELISPOT assays for detection of T cell immunity. As shown in **Figure 7**, we optimized several parameters of the ELISPOT assay and have developed a working ELISPOT assay (see Table 3, below). This will allow us to more sensitively quantitate T cell responsiveness to tumor antigens. In re-evaluating specific aim 3, we have determined that induction of an immune response to human tumor antigens in a mouse model may not accurately reflect the ability to that antigen to elicit immunity in humans. Thus, we plan to modify our method for evaluating the potential for eliciting anti-TAA immune responses by performing *in vitro* ELISPOT assays to assess induction of an immune response in using human cells. Recent experience in our laboratory, as well as reports in the literature, suggest that this may be a more reasonable method of determining the capacity of a human tumor antigen to elicit T cell responses. These studies will be based on published methods (10) that are now being used by our group in conjunction with the Immune Response Analysis Laboratory at UAB.

Table 3. Time course of γ IFN ELISPOT assay.
Normal donor peripheral blood mononuclear cells (PBMC) were cultured for the indicated number of days with either tetanus toxoid or irradiated autologous EBV-transformed cell lines. Control cultures were PBMC without antigens. Cultures were harvested, cells washed and plated at varying densities in antibody-coated γIFN ELISPOT plates for overnight incubation.

**γIFN positive cells/million (± SD)**

| donor | antigen | day 3 | day 4 | day 5 | day 6 |
|---|---|---|---|---|---|
| KA | none | <5 | <5 | <5 | <5 |
| | tetanus | 87+28 | 481+176 | 524+254 | 787+224 |
| DA | none | <5 | <5 | <5 | <5 |
| | tetanus | 58+11 | 237+37 | 162+83 | 255+38 |
| KJ | none | <5 | <5 | <5 | <5 |
| | tetanus | 50+18 | 124+30 | 97+21 | 163+21 |
| DS | none | 82+44 | 49+9 | 14 | 47+24 |
| | EBV | 8,691+2,096 | 1,956+278 | 472+36 | 1,240+424 |
| CJ | none | <5 | 10 | <5 | <5 |
| | EBV | 6,688+2,304 | 2,861+930 | 630+157 | 2,709+1,454 |

## KEY RESEARCH ACCOMPLISHMENTS

1. SEREX was validated in a mouse model of adenocarcinoma: Two tumor antigens have been identified and characterized at the molecular level (manuscript in press, appendix).

2. Polynucleotide immunization studies indicate that tolerance to these two antigens can be broken using this approach; however, antitumor immunity was not achieved using this vaccine strategy. This suggests that a modified strategy will be needed for effective antitumor immunization in breast cancer patients.

3. SEREX screening of a human breast cancer-derived cDNA libraries has identified a candidate tumor antigen. Molecular characterization of this tumor antigens is partially completed.

## REPORTABLE OUTCOMES:

### Manuscripts, Abstracts and Presentations

1. Manuscript in press:

Hampton TA, Conry RM, Khazaeli MB, Shaw DR, Curiel DT, LoBuglio AF, Strong TV. SEREX analysis for tumor antigen identification in a mouse model of adenocarcinoma. Cancer Gene Ther 1999 (accepted)

2. Abstracts and Presentations:

Strong TV, Guerrero A, Hampton TA, Conry RM, Ruppert JM, Curiel DT, LoBuglio AF. Serological identification of human breast tumor associated antigens by recombinant expression cloning. American Association of Cancer Researchers 39:262, 1998.

Hampton TA, Conry RM, Sumerel L, Khazaeli MB, Curiel DT, LoBuglio AF, Strong TV. The murine endogenous leukemia proviral envelope protein acts as a tumor antigen in MC38 cells. Gordon Research Conference on Cancer Molecular Biology, August, 1998.

### Degree Obtained:

1. The principal investigator, Tracy Hampton, has received her Ph.D. based on this work. (A petition for another graduate student, Alicia Sanders, to continue this research has been sent to the Grants Officer under separate cover).

### Informatics:

Upon delineation of the open reading frame, the S3.2 clone will be added to the list of putative tumor antigens catalogued in The SEREX Database, sponsored by the Ludwig Institute for Cancer Research (http.//www.licr.org/SEREX.html)

### Funding:

Feng Johanning is principal investigator on a recently funded grant from the American Cancer Society to explore endogenous retroviral proteins as tumor antigens. This grant was based on our experiments in a mouse model of adenocarcinoma as well as collaborative studies examining expression of the envelope gene in human breast cancer specimens (manuscript in preparation).

### Employment/Training

Tracy Hampton received her Ph.D. and has, as a result of this work, been hired as a Postdoctoral Fellow at Stanford University and successfully competed for a position on an NIH-funded training grant there.

## CONCLUSIONS

We have demonstrated that in a mouse model of adenocarcinoma, SEREX analysis offers a comparatively rapid means of identifying immunogenic tumor proteins. In the mouse system we identified two tumor antigens and characterized them at the molecular level. One of these antigens, the envelope protein of the endogenous ecotropic retrovirus has been shown to be a tumor antigen in other model systems. We demonstrated that in the MC38 syngeneic model, the expressed sequence is not mutated with respect to the germline sequence. Thus this protein is immunogenic based on its expression in tumor tissue and lack of expression in normal adult tissue. We also showed that this protein is likely to be a shared tumor antigen in several models of mouse tumors. This observation has led us to explore the possibility of human endogenous retroviral proteins acting as tumor antigens in breast cancer; and studies to test this hypothesis have been initiated and were funded by another agency. We have also identified a nuclear protein involved in regulation of gene expression as a potential antigen in the mouse model. Our studies have allowed us to recognize that polynucleotide immunization, while capable of breaking tolerance, will need to be optimized for elicitation of antitumor immunity. Additional strategies to more effectively elicit an immune response against self antigens are in development outside the scope of the current grant; however, we anticipate these studies will facilitate the translation of novel breast tumor antigens characterized herein into effective cancer vaccines for women with breast cancer.

We have extended the animal studies to the human have identified a potential breast tumor antigen. Our plan is to continue with the molecular characterization of this antigen as outlined in the original proposal. We will also perform more extensive analysis of the immune response elicited by this antigen and the relevance of that immune response to breast cancer. These studies will allow us to determine if this protein may be useful for diagnostic testing, staging and/or as a target for immunotherapy of this disease.

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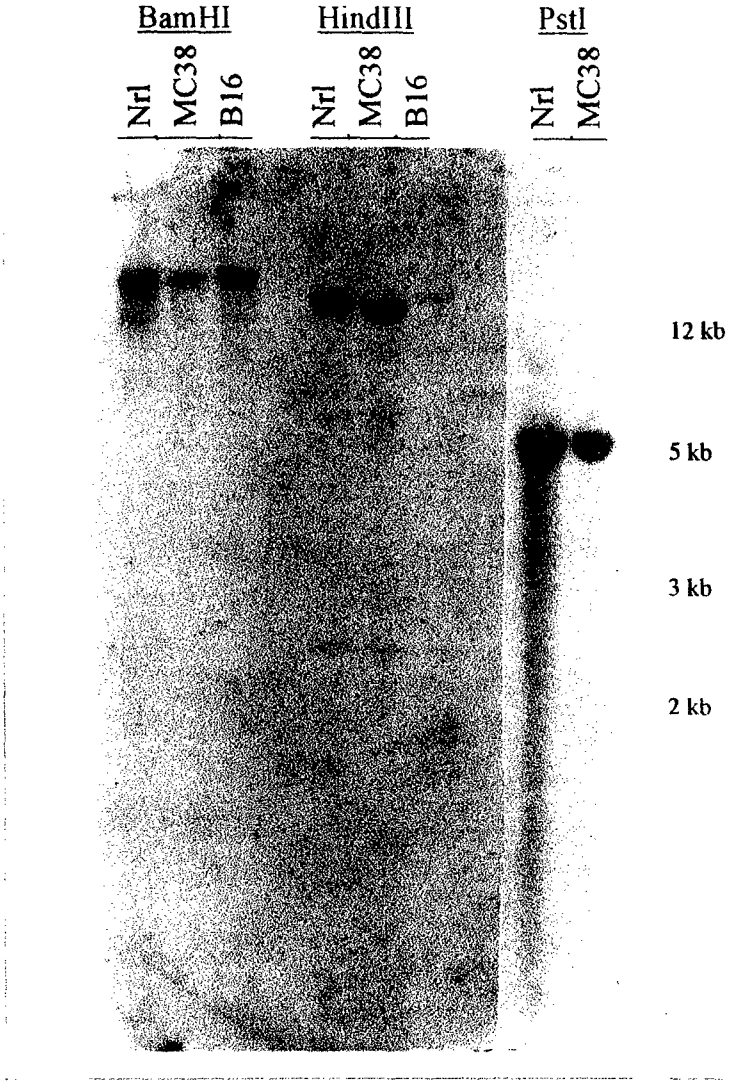



**Figure 1. Southern blot analysis of the ATRX gene.** Genomic DNA was isolated from normal colonic epithelium, MC38 cells, or B16 cells and digested with the indicated restriction enzyme. The ATRX specific probe was labeled with 32-P and used for hybridization. No rearrangements or amplification of the gene was demonstrated in tumor cells compared to normal DNA.

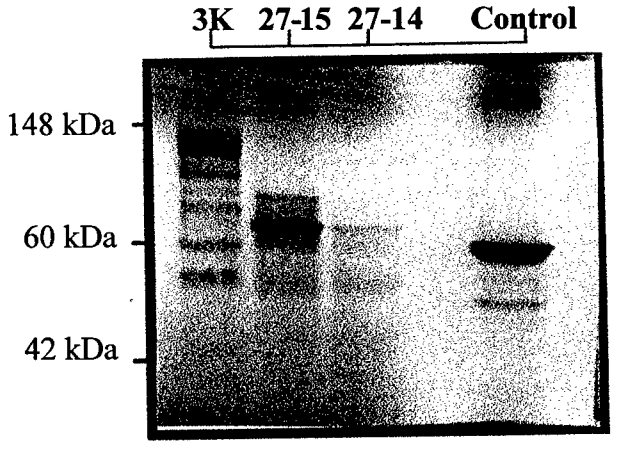



**Figure 2. Validation of protein production from plasmid DNA prior to polynucleotide immunization.** *In vitro* transcription and translation reactions were performed to validate expression from DNA constructs encoding the full-length envelope protein (clones 27-15 and 27-14) and the partial ATRX protein (clone 3K). Control DNA generates a protein of 61 kD.

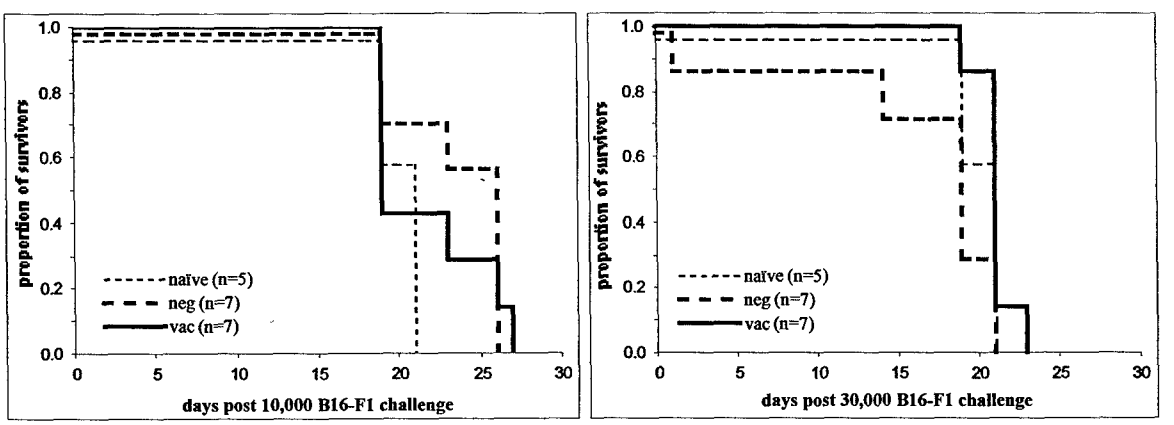



**Figure 3. Polynucleotide immunization with plasmid DNA encoding tumor antigens did not protect mice from tumor challenge.** Mice were immunized a mix of plasmid DNAs encoding the partial envelope sequence initially identified (1.3 kb), the full length envelope cDNA (3.0 kb) and the partial ATRX cDNA clone (2.8 kb). Survival curves from mice challenged with 10,000 or 30,000 syngeneic B16 cells indicates no difference between immunized mice, naive mice, or mice immunized with control plasmid DNA (no insert).

Methods: Mice were immunized intramuscularly with an equal mix of the antigen encoding plasmids (60 μg each injection), or with control plasmids. Mice were immunized on days 1, 22, 43, and 57. All mice received intraperitoneal injections of $1.6 \times 10^3$ international units of recombinant IL-2 daily for 5 consecutive days beginning 3 days after the first 3 DNA injections. After the immunization schedule was complete, mice were challenged subcutaneously with the indicated number of cells into the flank.

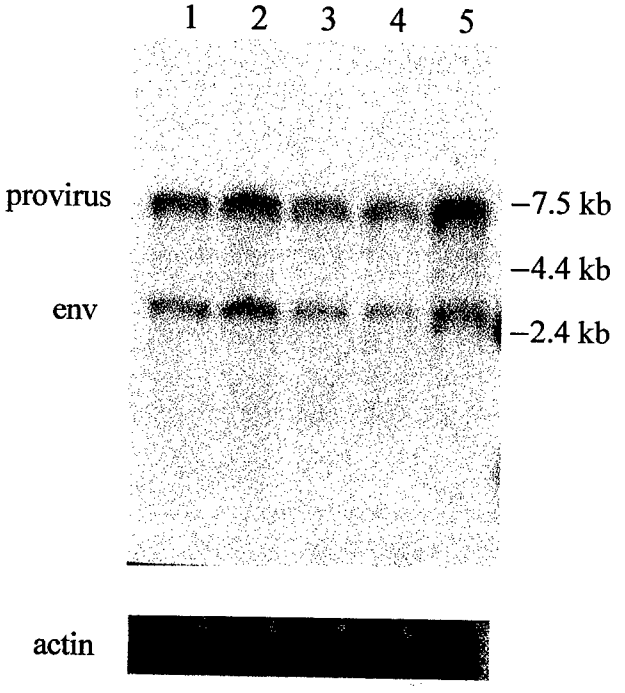



**Figure 4. Tumors developing in immunized mice maintained expression of the *env* transcript.** Tumors were excised from mice in each immunized group (as per Fig. 3), and RNA extracted for Northern blot analysis. All groups expressed high levels of the endogenous proviral and spliced *env* transcripts. Lanes 1 and 2: RNA from tumors grown in animals immunized with TAAs (including env) and receiving IL-2; Lane 3: RNA from tumors grown in mice immunized with control DNA and receiving IL-2; Lane 4 RNA from a tumor grown in a naive mouse; Lane 5 B16 cell line-derived RNA.

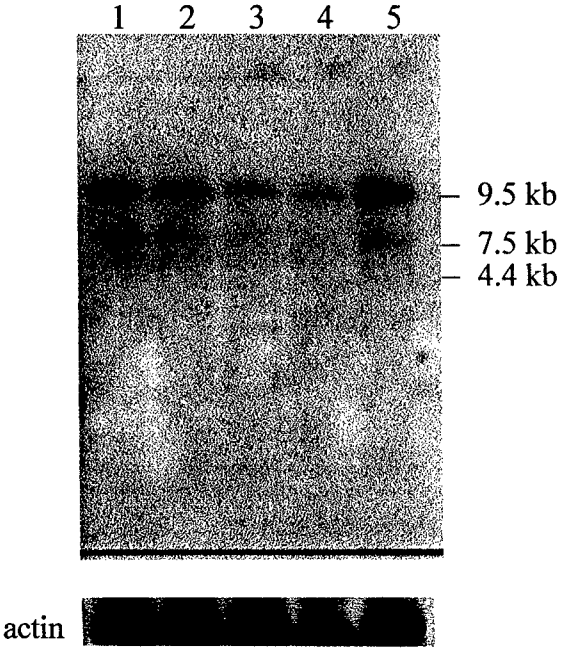
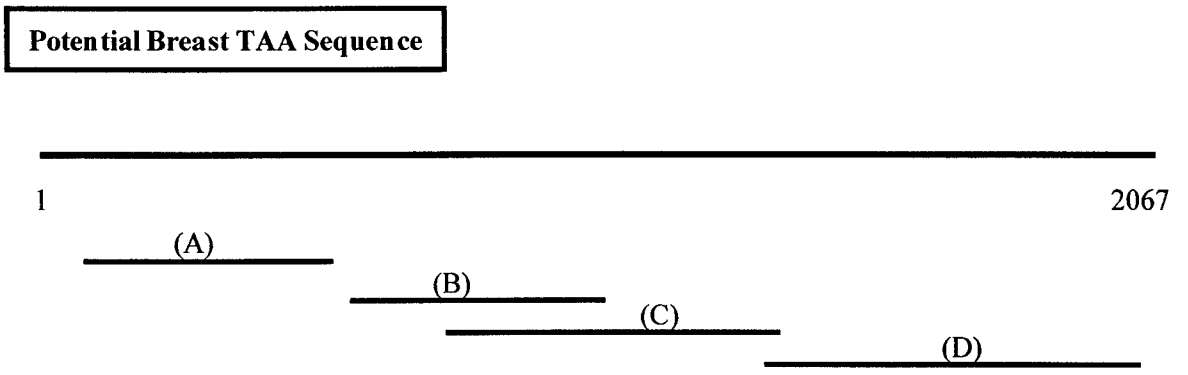



**Figure 5. Tumors developing in immunized mice maintained expression of *ATRX* mRNA.** Tumors were excised from mice in each immunized group (as per Fig. 3), and RNA extracted for Northern blot analysis. All groups expressed moderate levels of the *ATRX* transcript. Lanes 1 and 2: RNA from tumors growing in animals immunized with TAAs (including *ATRX*) and receiving IL-2; Lane 3: RNA from tumors grown in mice immunized with control DNA and receiving IL-2; Lane 4 RNA from a tumor grown in a naive mouse; Lane 5 B16 cell line-derived RNA.

[redacted]




(A) 96% (371/384)
AA411639 519 bp mRNA EST
**Homo sapiens cDNA clone similar to bovine ubiquinone-oxidoreductase subunit**

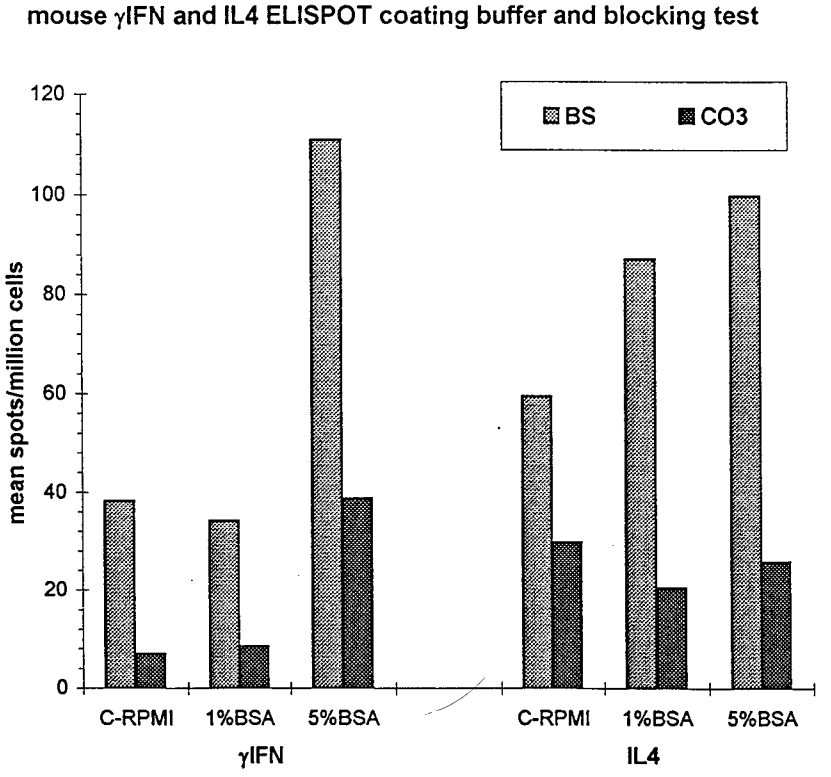
(B) 99% (321/323)
AA429219 421 bp mRNA EST **Total fetus Homo sapiens cDNA clone 759807**

(C) 94% (442/469)
H09067 477 bp mRNA EST **Infant brain Homo sapiens cDNA clone 46156**

(D) 97% (498/509)
AA075427 514 bp mRNA EST **Ovarian cancer Homo sapiens cDNA clone 544880**

**Figure 6. Sequence analysis of S3.2 (2.1 kb insert) demonstrated homology with various ESTs.** These sequences have unknown function and chromosomal location.

ELISPOTmo_9_1.xls rev. 9/4/98




| | | |
|---|---|---|
| ***Coating buffers:*** | Borate saline, pH 8.4<br>Carbonate buffer, pH 9.8 | |
| ***Blocking buffers:*** | **C-RPMI** | RPMI-1640 + 10% FBS |
| | **5%BSA** | 5% (w/v) BSA in PBS |
| | **1%BSA** | 1% BSA (w/v) in borate saline |
| ***Protocol:*** | C57BL6 mouse splenocytes stimulated in-plate bulk culture for 2 days with or without 1:100 PWM. | |

**Figure 7. Optimization of ELISPOT assay.** As part of the optimization of the ELISPOT protocol, coating buffers and blocking buffers were evaluated. C57BL6 mouse splenocytes were stimulated for 48 hours in bulk culture with the poke weed mitogen. After incubation, the cells were plated into anti-γIFN or anti-IL4 coated plates, and ELISPOTS were developed approximately 48 hours later.

# SEREX Analysis for Tumor Antigen Identification in a Mouse Model of Adenocarcinoma

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## ABSTRACT

Evaluation of immunotherapy strategies in mouse models of carcinoma is hampered by the limited number of known murine tumor antigens. Although tumor antigens can be identified based on cytotoxic T cell activation, this approach is not readily accomplished for many tumor types. We applied an alternative strategy based on humoral immune response, SEREX, to the identification of tumor antigens in the murine colon adenocarcinoma cell line, MC38. Immunization of syngeneic C57BL/6 mice with MC38 cells by three different methods induced a protective immune response with concomitant production of anti-MC38 antibodies. Immunoscreening of an MC38-derived expression library resulted in the identification of the endogenous ecotropic leukemia virus envelope (env) protein and the murine ATRX protein as candidate tumor antigens. Northern blot analysis demonstrated high levels of expression of the *env* transcript in MC38 cells and several other murine tumor cell lines, while expression in normal colonic epithelium was absent. *ATRX* was found to be variably expressed in tumor cell lines and normal tissue. Further analysis of the expressed *env* sequence indicated that it represents a nonmutated tumor antigen. Polynucleotide immunization with DNA encoding the env polypeptide resulted in strong and specific antibody responses to this self antigen in all immunized mice. Thus SEREX offers a rapid means of identifying tumor antigens in murine cancer models.

## INTRODUCTION

The concept of cancer immunotherapy is predicated on the existence of tumor-associated antigens (TAAs) against which the host is capable of mounting an immune response leading to tumor rejection. To this end, immune responses in tumor-bearing individuals have been described for a variety of mutant tumor antigens such as mutant p53 (1-3) and ras (4-7), as well as nonmutant tumor proteins including erbB-2 (8, 9), MART-1 (10, 11), MAGE-1 (12, 13), tyrosinase (14, 15), and MUC-1 (16). Such tumor-specific responses suggest that the immune system may be amenable to strategies that further induce or enhance patients' immune responses to their own tumor cells. Important to the concept of developing effective immunotherapies is the ability to break immunological tolerance to self antigens. However, the ability to evaluate approaches for breaking tolerance to specific antigens in mouse models of cancer is limited by the fact that few murine tumor antigens have been described to date. Accordingly, many murine immunotherapy models utilize heterologous antigens (for example, 17-19), which may not address issues critical to the development of effective immunotherapeutic strategies. A more complete understanding of the spectrum of murine antigens capable of eliciting anti-tumor immune responses is needed to better understand the interaction of cancer and the immune system as well as for the development of more effective cancer vaccines.

Several methods have been employed to isolate and clone TAAs. Due to the critical role of cytolytic T cells (CTLs) in mediating antitumor immunity, these approaches have largely consisted of the isolation of antigens recognized by CTLs which have been expanded *in vitro*. Tumor antigens have been identified in this manner by acid elution of antigenic peptides bound to major histocompatibility complex class I molecules (20). A second, genetic, approach assays CTL responsiveness to target cells transfected with cDNA libraries to identify immunogenic peptides (21, 22). While these approaches have proven valuable for TAA identification in some tumor types, particularly human melanoma, widespread application is hampered by difficulties in establishing long-term CTL cultures. In addition, it has not yet been established that CTLs generated *in vitro* are representative of the tumor-specific CTLs *in vivo*.

Thus, complementary approaches for the identification of novel immunogenic tumor antigens are warranted.

The activation of CTL responses is dependent on T-helper cells and may be accompanied by the induction of humoral immunity. Indeed, humoral immune responses to known TAAs have been described in tumor-bearing individuals (1, 2, 23-27). The presence of antibodies directed towards known tumor antigens suggests its use for the identification of novel TAAs, and defines the antigens as immunogenic. The feasibility of such a strategy for identifying tumor antigens was demonstrated by Pfreundschuh and coworkers, who identified both known and novel tumor antigens using autologous sera from cancer patients to screen tumor-derived expression libraries (28), an approach they termed "serological identification of antigens by recombinant expression cloning" (SEREX). This technique has succeeded in greatly expanding the scope of human tumor antigens (29). By comparison, the catalogue of cloned mouse tumor antigens remains quite small.

Mouse models of syngeneic tumors provide a useful system in which to investigate the nature of TAAs. The MC38 colonic adenocarcinoma cell line was derived from C57BL/6 mice treated with the carcinogen 1,2-dimethylhydrazine (30). These cells produce tumors when injected subcutaneously in syngenic mice, but immunization of naive syngeneic mice with MC38 cells can induce an immune response which mediates tumor rejection upon challenge with an otherwise lethal dose of live cells. Thus, these cells express cryptic tumor antigens which stimulate antitumor immune responses *in vivo*. Here we describe the application of SEREX technology towards the identification of MC38 antigens. This approach identified the endogenous ecotropic retroviral envelope and the murine ATRX proteins as putative tumor antigens in these cells. These antigens were reactive with sera from mice immunized with MC38 cells in three different ways. The env protein was found to be a nonmutant tumor antigen, and polynucleotide immunization was utilized to demonstrate that an immune response could be elicited to this antigen. These studies demonstrate the utility of SEREX as a means to rapidly identify murine tumor antigens for further evaluation in immunotherapy models.

## MATERIALS AND METHODS

### Cell Lines and Culture Conditions

MC38 and MC38-CEA-2 (31) cells were kindly provided by Dr. Steven Rosenberg at the National Cancer Institute and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). B16 is a mouse melanoma cell line which arose spontaneously in C57BL/6 mice (32). These cells were maintained in Minimal Essential Medium-Eagle's (EMEM) with 10% FCS. The mouse lymphoma cell line EL4 (33), a chemically induced line derived from a C57BL/6 mouse, was maintained in DMEM supplemented with 10% horse serum. The mouse mammary tumor cell lines 4T01 and 4T07 are sublines of a spontaneously arising mammary tumor in a BALB/cf$C_3$H mouse (34), and were maintained in DMEM/F12 supplemented with 10% FCS. CT26 cells are colonic adenocarcinoma cells from a BALB/c mouse induced by N-nitroso-N-methylurethane (35), and they were cultured in RPMI 1640 with 10% FCS. P815, a mastocytoma line derived from a DBA/2 mouse (36), was maintained in RPMI 1640 with 10% FCS; and C2C12, a mouse myoblast cell line (ATCC), was maintained in DMEM with 10% FCS.

### Immunization Protocol

To elicit anti-MC38 immune responses, groups of twelve mice were immunized in one of three ways. Group I mice were injected with 5 x $10^5$ MC38 cells into the right foot pad and tumors were excised 10-14 days later. Ten of twelve mice survived the amputation and were available for challenge on day 29 with 3x$10^5$ cells injected subcutaneously into the flank. Eight of the ten mice rejected the day 29 tumor challenge and received a second identical tumor challenge on day 79, followed 14 days later by exsanguination to harvest sera. Group II mice were injected subcutaneously on days 1 and 15 with 1 x $10^6$ MC38 cells which had been irradiated with 15,000 cGy. Mice were then challenged with 3 x $10^5$ non-irradiated tumor cells on day 29. Three of 12 animals developed tumors and were sacrificed. The remaining 9 animals were re-challenged with 3 x $10^5$ non-irradiated tumor cells on day 54. Sera were collected from these animals 14 days later. Group III animals were immunized with a plasmid DNA

encoding human carcinoembryonic antigen (CEA) and challenged with MC38 cells stably transfected to express human CEA (MC38-CEA-2, 31). We have previously shown that polynucleotide immunization with a CEA-encoding plasmid results in reliable rejection of MC38-CEA-2 cells (37). Thus, 12 mice received intramuscular injections of 50 μg of plasmid encoding CEA on days 1 and 15 followed by tumor challenge with 3 x $10^5$ MC38-CEA-2 cells on days 29, 41, and 54. Eight of twelve mice successfully rejected all three challenges with MC38-CEA-2 cells, and sera were collected from these mice 14 days after the last challenge. In each of the three immunization experiments described above, tumors grew in twelve of twelve naive control mice, validating each challenge with MC38 or MC38-CEA-2 tumor cells.

**Assay for Antibody Response to Intact Tumor Cells**

To determine whether the various immunization strategies had elicited an antibody response to MC38 tumor cells, a cell binding assay was performed as previously described (38). Briefly, tumor cells were washed with phosphate buffered saline (PBS) and 5 x $10^5$ cells were aliquoted per tube in 100 μl of PBE (PBS with 1% bovine serum albumin). Each tube then received 100 μl of a 1:10 dilution of immune sera or normal mouse sera in PBE, and the cell/serum mixture was incubated at room temperature for one hour on an orbital shaker. Cells were then washed with 4 mls of PBS to remove unbound antibody. After washing, the cells were resuspended in 100 μl of PBS containing 0.1% pigskin gelatin and 100 ng of $^{125}$I-labeled Staphylococcus aureus protein A (approximately 200,000 cpm per tube), and incubated for one hour at room temperature with shaking. Staphylococcus protein A (SPA) was obtained from Sigma Chemical Company and was radiolabeled by the Iodogen method, Pierce Chemical Company. The cells were washed with 4 mls of PBS and counted in a gamma scintillation counter. The specific activity of the $^{125}$I-labeled SPA was used to convert cpm bound to number of molecules bound per cell (38). The SPA binding to cells incubated in PBE only (no serum) was subtracted as non-specific binding. MC38-CEA-2 cells incubated with CEA-specific monoclonal antibody (COL-1) served as a positive control.

## cDNA Library Construction and Immunoscreening

Total RNA from MC38 cells or MC38-CEA-2 cells was isolated using RNA Stat 60 RNA isolation reagent (Tel-Test B), and mRNA was isolated on oligo-dT beads (PolyA-Quik mRNA Isolation Kit, Stratagene). A cDNA library was constructed in the Zap Express vector (Stratagene), which is capable of directing expression in both prokaryotic and eukaryotic hosts. Briefly, mRNA was reverse transcribed using an oligo dT primer with an internal *Xho*I site as well as a random primers which included a *Xho*I restriction site at the end. The reverse transcription reaction included 5-methyl dCTP. After second strand synthesis, *Eco*RI adapters were added by ligation and the cDNA was restricted with *Xho*I. cDNA fragments were cloned into the directional λZapExpress vector, packaged and used to infect *E.coli* cells. As a preliminary characterization of the library, inserts from random plaques were amplified by the polymerase chain reaction (PCR) and demonstrated insert sizes ranging from approximately 300 bp to 3.5 kb with greater than 90% recombinants (not shown).

For immunoscreening, recombinant plaques were plated at a density of approximately 20,000 plaques per 150 mm plate, and protein expression was induced using nitrocellulose filters saturated with isopropyl b-D-thioglactoside (IPTG). Filters were lifted, blocked with 1% BSA in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl) and screened with sera from mice immunized as above to detect reactive plaques. Primary mouse sera were preabsorped with *E. coli* phage lysate (Stratagene) and diluted 1:250 for screening. After incubating filters with diluted mouse sera, the filters were washed with TBST (TBS with 0.05% Tween 20 [Sigma]) and incubated with alkaline phosphate-conjugated goat anti-mouse IgG antibodies (Stratagene) at a dilution of 1:5,000 for one hour at room temperature. After washing, an NBT/BCIP colorimetric substrate was used to identify positive clones. Positive plaques were purified to clonality for further study.

## DNA Sequencing

Phagemid DNA from positive plaques was rescued by *in vivo* excision using a helper phage system (Stratagene). Purified DNA was sequenced by dideoxy sequencing using Sequenase 2.0 and $^{35}$-S

labeled dATP (USB). Approximately 250 basepairs of sequence was determined at both the 5' and 3' ends using T3 and T7 primers, respectively. Sequences were searched against the National Center for Biotechnology (NCBI) databases using the BLASTN program.

To sequence the *env* gene expressed in MC38 for comparison with C57BL/6 genomic sequences, PCR products were generated from reverse transcribed MC38 total RNA and C57BL/6 genomic DNA. The 2.6 kb coding region was amplified using the following primers:

5' GAGAAAAGCTTCGCCCCCGATAAACCATGGAG 3'

5' GCGCAAGCTTTGCACCAGCAAAAGGCTTTATT 3'

*Hin*dIII sites are underlined. Sequencing of the PCR products was performed using an IBI automated sequencer with the PCR primers as well as internal sequencing primers.

**Northern Blot Analysis**

Total RNA was isolated from cultured cells using RNA Stat-60 (Tel-Test B) according to the manufacturer's directions. To isolate RNA from colonic epithelium, a section of colon was isolated, opened longitudinally, and the epithelium was recovered by gentle scraping. Epithelial cells were homogenized in RNA Stat-60 and processed as for the cultured cells. Approximately eight micrograms of total RNA from each sample were electrophoresed on a 1% denaturing agarose gel and transferred to a nylon membrane (Amersham). The blot was prehybridized for 2 hours at 65°C in a solution of 6xSSC, 5x Denhardt's, 100µg/ml sheared salmon sperm DNA, 1 mM EDTA and 0.5% SDS. For analysis of *env* expression, two hundred ng of a random-primed $^{32}$-PdCTP labeled probe was generated corresponding to bp 6004 - 6543 of the *env* gene, a *Bgl*II to *Bam*HI fragment. This probe recognizes only the ecotropic *env* sequence (39, 40). For *ATRX*, the 2.8 kb insert was isolated and labeled. Each probe was hybridized overnight in hybridization solution as described above, with the probe at a concentration of 20 ng/ml. After hybridization, the blot was washed successively in 2xSSC with 0.5 % SDS, 2x SSC with 0.1% SDS, and 0.1x SSC with 0.1% SDS; and the blot was autoradiographed at

-70°C for a period of 1 to 4 days. To control for loading differences, the blot was stripped with boiling 0.1% SDS and rehybridized with a probe specific for the mouse beta-actin gene (Stratagene).

**Southern Blot Analysis**

For Southern blot analysis, genomic DNA from MC38 cells, B16 cells, and normal colonic epithelial cells was isolated by lysis in 50 mM Tris, pH8, 10 mM EDTA, 10 mM NaCl, 1% SDS and treatment with 100μg/ml Proteinase K overnight at 50°C. Following phenol/chloroform extraction, the DNA was precipitated with ethanol and resuspended in 10mM Tris, 1 mM EDTA, pH 8.0. Approximately 10 μg of DNA was subjected to digestion with the appropriate restriction enzyme, electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Amersham). The membrane was hybridized with an *env*- or *ATRX*- specific probe as above.

**Polynucleotide Immunization and Isotype Analysis**

Groups of 10 mice were immunized with DNA encoding the 1.3kb partial *env* cDNA or with vector backbone. Intramuscular injections of 50 μg of plasmid DNA (at 1 mg/ml) were made on days 1, 15, and 29. Sera were collected 4 days after the third immunization for analysis of env-specific antibody via screening against the env-expressing plaques as described above. For isotype analysis, alkaline phosphatase conjugated, isotype specific antibodies (Clonotyping System, Southern Biotechnology Associates), were used as secondary antibodies at a dilution of 1:1,000.

## RESULTS

### Immunization and Detection of Antibodies Directed Against MC38 Antigens

Three groups of mice were immunized to generate sera reactive with MC38 cells. Group I mice were injected with MC38 cells with subsequent tumor excision, and group II animals were immunized with irradiated MC38 cells. Both of these groups were then challenged with live MC38 cells, which were rejected by most animals (17 of 22). A third group of animals was immunized with plasmid DNA encoding human CEA (pCEA) and challenged with MC38 cells expressing CEA, as previously reported (37). Eight of twelve animals in this group rejected three consecutive tumor challenges. After the immunization schedules were completed, the presence of MC38-reactive antibodies was detected in an MC38 cell binding assay (Fig. 1). All three groups of immunized mice demonstrated high levels of antibody binding to intact MC38 cells, whereas naive mouse serum did not. Mice immunized with pCEA and challenged with MC38-CEA-2 cells produced antibodies to the parental MC38 cells in addition to CEA-specific antibodies, as manifested by increased antibody binding to MC38-CEA-2 cells compared to parental MC38 cells. Thus, these serum samples contained antibodies useful for detection of cryptic tumor-associated antigens in MC38 cells.

### Identification of Reactive Plaques

Sera from immunized animals (Group III) were used to screen an MC38-derived cDNA library. Reactive plaques were then purified to homogeneity in secondary and tertiary screens. Screening of approximately $10^6$ plaques resulted in the identification of two independent positive plaques. Amplification of the insert of one of these plaques using the polymerase chain reaction demonstrated the presence of a 1.3 kb insert. Importantly, sera from animals immunized with MC38 cells by all three routes described above (Groups I, II and III) were strongly reactive with the 1.3 kb plaque (not shown). Isotype analysis revealed that reactive antibodies were predominantly $IgG_{2a}$ and $IgG_{2b}$, with IgM and $IgG_1$ being weakly reactive. Dideoxy sequencing was performed on rescued plasmid DNA using the T3 and T7 primers present in the vector. A search of the NCBI database revealed identity of the clone with a

portion of the endogenous ecotropic leukemia proviral sequence (MuLV). This insert sequence includes the 3' portion of the envelope protein sequence (*env*) as well as the 3'LTR (Genbank accession numbers J01998 and U63133) (Fig 2). The clone contains basepairs 7071 to 8374 of the published sequence (41), corresponding to amino acids 430 to the C-terminus of the envelope protein. The env polypeptide encoded by this clone includes the last one-third of the extracellular portion of the protein, as well as the entire transmembrane domain. A 99 bp deletion of the 3' LTR was also noted, as discussed below.

The second plaque had an insert size of approximately 2.8 kb in size and was also reactive with all groups of MC38-immunized animals. Sequence analysis of the insert revealed that it represents a portion (bp 503-3327) of the murine homologue of the *ATRX* gene (accession #AF026032). This gene encodes a protein with potential DNA binding and helicase activity (42, 43). The sequence of the 2.8 kb fragment derived from MC38 mRNA was not mutated compared to the published sequence.

**Assessment of *env* and ATRX mRNA Expression in Murine Tumor Cells**

Northern blot analysis was performed to determine if the *env* and *ATRX* genes are actively transcribed in MC38 cells (Fig. 3). Total RNA was isolated from MC38 cells and several other murine tumor cell lines. These included the C57BL/6 derived cell lines B16 (melanoma) and EL4 (lymphoma), as well as the BALB/c-derived colonic line CT26, and mammary tumor cell lines 4T1 and 4T07. The mastocytoma cell line P815 from DBA/2 mice was also analyzed, as was the nontransformed myoblast cell line C2C12. Total RNA was also isolated from the normal colonic epithelium of a C57BL/6 mouse. The endogenous ecotropic *env* gene was previously implicated as a melanoma-specific antigen in B16 cells (44). High levels of *env* gene expression were detected in MC38, B16, CT26 and 4T07 cells (Fig 3A). Expression was also noted in 4T1 and EL4, with very low levels of expression in P815 cells. Expression of the proviral sequences was not detected in the myoblast line C2C12 nor in normal colonic epithelium. Thus, while not expressed in normal cells, the ecotropic endogenous retrovirus is expressed in a variety of murine tumor cell lines, including both spontaneously arising (B16, 4T1, and 4T07) and carcinogen-induced tumors (MC38, EL-4, P815, and CT26). The *ATRX* gene was found to be

expressed in all cell lines tested, as well as in normal colonic epithelium (Fig 3B). Expression was strongest in the tumor cell lines B16 and EL4.

**Southern Blot Analysis**

We next investigated the genomic structure of the env-encoding proviral sequence in MC38 cells. Endogenous retroviruses may become activated as the result of a point mutation and remain single copy (45), or activation may be accompanied by amplification or rearrangement of the proviral sequence (46). C57BL/6 cells normally contain a single copy of the endogenous ecotropic proviral sequence, which resides on chromosome 8 (47). To determine if this MuLV proviral sequence was amplified and/or rearranged in MC38 cells, Southern blot analysis was performed. Genomic DNA from normal colon, MC38 cells and B16 cells was digested with the restriction enzymes *Bam*HI, *Hind*III, *Pst*I, *Pvu*II, and *Kpn*I . Hybridization of the DNA with the ecotropic-specific *env* probe revealed the presence of novel fragments in the MC38 DNA, not found in the normal DNA and in some cases distinct from those of B16 DNA (Fig 4). This suggests that the MC38 proviral sequence has likely undergone amplification and reintegration into new genomic sites. Southern blot analysis of the *ATRX* gene revealed no apparent genomic rearrangements in MC38 or B16 cells compared to normal genomic DNA (data not shown).

**Analysis of the *env* sequence**

We chose to further study the envelope protein as a tumor antigen in this mouse model in order to determine the basis of its immunogenicity. To determine if the envelope sequence expressed in MC38 cells represents a mutant or nonmutant tumor antigen, we next sequenced the expressed *env* sequence and compared it to the published sequence, derived from the AKR mouse strain (41), and to the endogenous sequence in C57BL/6 genomic DNA. The full-length*env* cDNA was amplified from MC38 total RNA by polymerase chain reaction after reverse transcription. The normal sequence was amplified from genomic DNA extracted from a C57BL/6 mouse. Previously reported coding sequence differences between B16 *env* (also derived from a C57BL/6 mouse) and that of the AKV (44) were either not found in the MC38 expressed sequence (nucleotides 6116, 6419, 7017 and 7085; reference 41), or were found

to be the same in the MC38-derived *env* and the C57BL/6 genomic sequence. Thus the differences between the MC38 and AKR-derived sequences are likely to represent variations between the mouse strains. Also of note, as reported by others (44, 46), the *env* gene expressed in MC38 cells has a 99 bp deletion in the 3'LTR compared with AKV; however, this change also appeared to be present in the C57BL/6 genomic sequences when size was examined by PCR (data not shown).

**Polynucleotide Immunization**

As preliminary and rapid means to assess the potential of the envelope polypeptide to act as a immunogenic tumor antigen, the 1.3 kb insert was rescued as phagemid DNA from the reactive plaque and used for polynucleotide immunization. Mice were immunized with the *env*-encoding DNA, and antibody production was subsequently measured. Intramuscular immunization of C57BL/6 mice with the nonmutant *env* sequence elicited a strong, specific anti-env antibody response in all immunized mice (Fig 5). By contrast, animals immunized with the vector alone exhibited no reactivity with the env protein. Isotype analysis of the anti-env antibody response revealed the presence of several isotypes including $IgG_1$, $IgG_{2a}$, and $IgG_{2b}$, suggesting the presence of both Th1 and Th2 type responses(48, 49).

## DISCUSSION

A limited number of murine tumor antigens have been described to date, and the ability of these antigens to elicit effective antitumor immunity is currently an area of active investigation. While prospects for the development of immunotherapeutic modalities have recently been improved by advances in the field of immunology (50, 51), knowledge of the spectrum of tumor antigens important in mediating immune interactions is needed. Additionally, it is important to catalogue the nature of murine tumor antigens, such that a comparison with human cancers can be explored and more appropriate animal models can be developed for evaluating immunotherapeutic approaches to cancer. We have used a strategy for tumor antigen identification which exploits the humoral immune responses that accompany induction of antitumor immunity, and identified the MuLV endogenous ecotropic *env* gene-encoded protein and the ATRX protein as candidate tumor antigens in MC38 colon adenocarcinoma cells.

The ATRX protein is expressed in a wide variety of normal tissues in mouse and human (52). This protein contains highly conserved domains which classify it as a member of the helicase/ATPase superfamily (53). It thought to be a nuclear protein involved in the regulation of gene expression (54, 55). In humans, a deficiency in this protein is associated with a severe mental retardation syndrome (ATR-X syndrome) which includes alpha-thalassemia, urogenital abnormalities, and a characteristic facial appearance. A role for *ATRX* in cancer has not yet been described. Northern blot analysis demonstrates that this gene is expressed to a variable degree in all murine tumor lines tested. No mutations were identified in the partial cDNA isolated; and additional studies are underway to determine the basis of this protein's immunogenicity as well as to evaluate the potential of ATRX to serve as a tumor antigen.

More is known about the role of the envelope protein as a potential tumor antigen. This protein is encoded by one of the numerous endogenous retroviral sequences present in the normal mouse genome. Endogenous retroviruses are relatively stable elements present in multiple copies throughout the genome

and inherited in a Mendelian fashion (56). C57BL/6 mice carry a single copy of an endogenous ecotropic virus, and it is defective and transcriptionally silent in normal tissues (44). Activation of endogenous retroviruses spontaneously or following exposure to carcinogens has been described in several mouse strains. Activation may occur by several mechanisms, including point mutation (45), transcomplementation or recombination with other endogenous retroviruses (57-59), or by amplification and rearrangement of the locus (46). Here activation of *env* expression in MC38 cells is associated with appearance of novel bands on Southern analysis, suggesting that amplification and reintegration events are likely to have occurred. The exact nature of the activating mutation(s) will require further study.

Although the env protein was identified in this study on the basis of humoral immunity, it has been determined to be a target of CTL activity in both C57BL/6 mice (44) and BALB/c mice (60). The identification of a CTL-activating protein by immunoscreening further supports the use of antibody-based screening for the identification of relevant tumor antigens. Further, while most murine tumor antigens are thought to be unique to a particular tumor (61, 62), our findings suggest that *env* is expressed in several murine tumors. This antigen is expressed in both spontaneous and mutagen-induced tumor cell lines derived from mice of different genetic background, and from a variety of tissue types (Fig 3A). Sequence analysis revealed no apparent differences between the sequences expressed in the MC38 cells and the genomic sequences of the *env* gene, suggesting that this antigen is a true "self" antigen. Thus, env is immunogenic based on its expression in the tumor. Polynucleotide immunization demonstrated that this protein is capable of acting as an immunogen in C57BL/6 mice. A specific IgG antibody response was elicited in all immunized mice. Interestingly, this antigen may also have relevance to human cancer. The human homologues of the *env* gene, contained in human endogenous retroviral elements (HERVs), may also act as tumor antigens. HERVs are also widely dispersed throughout the human genome. Unlike mice, however, expression of a few of these proviral sequences has been reported in some normal human tissues, most notably placenta (63). HERV expression has also been noted in a variety of tumor cell lines. Recently, antibodies to the HERVK10 env sequence

were reported to be present in a large percentage of patients with seminoma (64). These attributes may make this tumor antigen an ideal target for additional studies relevant to tumor vaccinology.

In summary, application of SEREX to mouse models of carcinoma offers a comparatively rapid means of identifying putative tumor antigens which can then be evaluated for utility in gene therapy approaches to cancer immunotherapy. The development of more accurate preclinical models for the evaluation of improved vaccination strategies is one important goal of such studies. This approach may also be important for expanding known human tumor antigens through the identification of murine homologues.

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* Acknowledgment will be changed to recommended form at galleys.

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## FIGURE LEGENDS

**Figure 1. Antibody response to intact tumor cells.** Sera collected from mice immunized as outlined in the text were examined for antibody binding to intact tumor cells. Antisera from naive mice or immunized mice (groups I-III) were incubated with MC38 cells. In addition, sera from naive mice and animals immunized in group III were incubated with MC38-CEA-2 cells. Results are reported as the number of molecules of antibody bound per cell, as described in Methods. Binding of a CEA-specific monoclonal antibody (COL-1) to MC38 cells stably expressing human CEA (MC38-CEA-2) served as positive control.

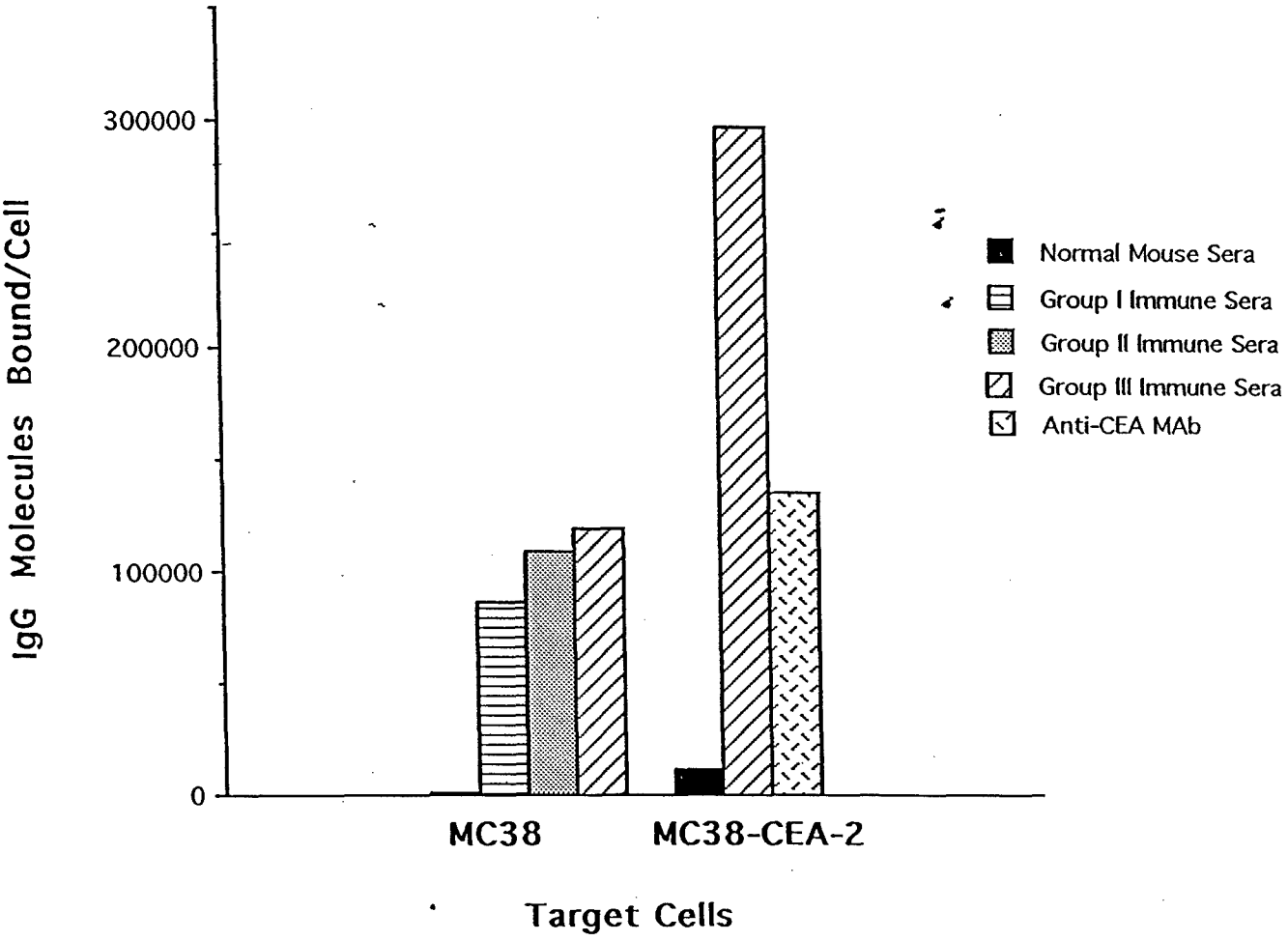
**Figure 2. Identification of a positive clone as the murine endogenous ecotropic retrovirus *env* gene.** Partial sequence analysis of the 1.3 kb clone, reactive with sera from all three groups of immunized mice, aligned with the sequence of the murine endogenous ecotropic retrovirus. Approximately 200 bp of the 5' end of the 1.3 kb clone is shown in alignment with the endogenous ecotropic murine leukemia virus *env* sequence acquired from Genbank (accession numbers: J01998).

**Figure 3. Northern blot analysis of *env* and *ATRX* gene expression in normal colonic epithelium and murine tumor cell lines.** A. Expression of the *env* gene was examined. Total RNA was hybridized with an envelope-specific probe. The lower transcript presumably represents the mature, spliced *env* transcript, while the upper band represents the full proviral transcript. No expression of the *env* gene was detected in normal colonic epithelium, even after prolonged autoradiography. The blot was stripped and rehybridized with a mouse beta-actin probe to demonstrate approximately equal loading (bottom panel). B. Expression of the *ATRX* gene was similarly examined. The full length transcript (approximately 10 kb) and an apparent alternatively spliced form were detected.

**Figure 4. Southern blot analysis of the endogenous ecotropic retrovirus in C57BL/6 genomic DNA, MC38 genomic DNA, and B16 genomic DNA.** A. Diagrammatic representation of restriction sites in the ecotropic endogenous proviral sequence. The location of the ecotropic-specific probe is indicated. B. Genomic DNA from C57BL/6 mouse colon (Nrl), MC38 cells and B16 cells was digested with the indicated restriction enzymes and hybridized with the ecotropic specific *env* probe. The arrows indicate the location of the endogenous proviral sequence in normal C57BL/6 genomic DNA.

**Figure 5. Induction of humoral immune response to the env protein following polynucleotide immunization.** Sera from four mice immunized with the 1.3 kb *env* cDNA (E1-E4) were incubated with filter lifts of phage encoding the env protein mixed with an approximately equal ratio of control, negative phage. Half of the plaques (encoding env) are strongly reactive, demonstrating the specificity of the reaction. Sera from mice immunized with an empty phagemid vector (N1-N4), were used for screening in the same manner, and did not contain antibodies which recognize the env protein.

Figure 1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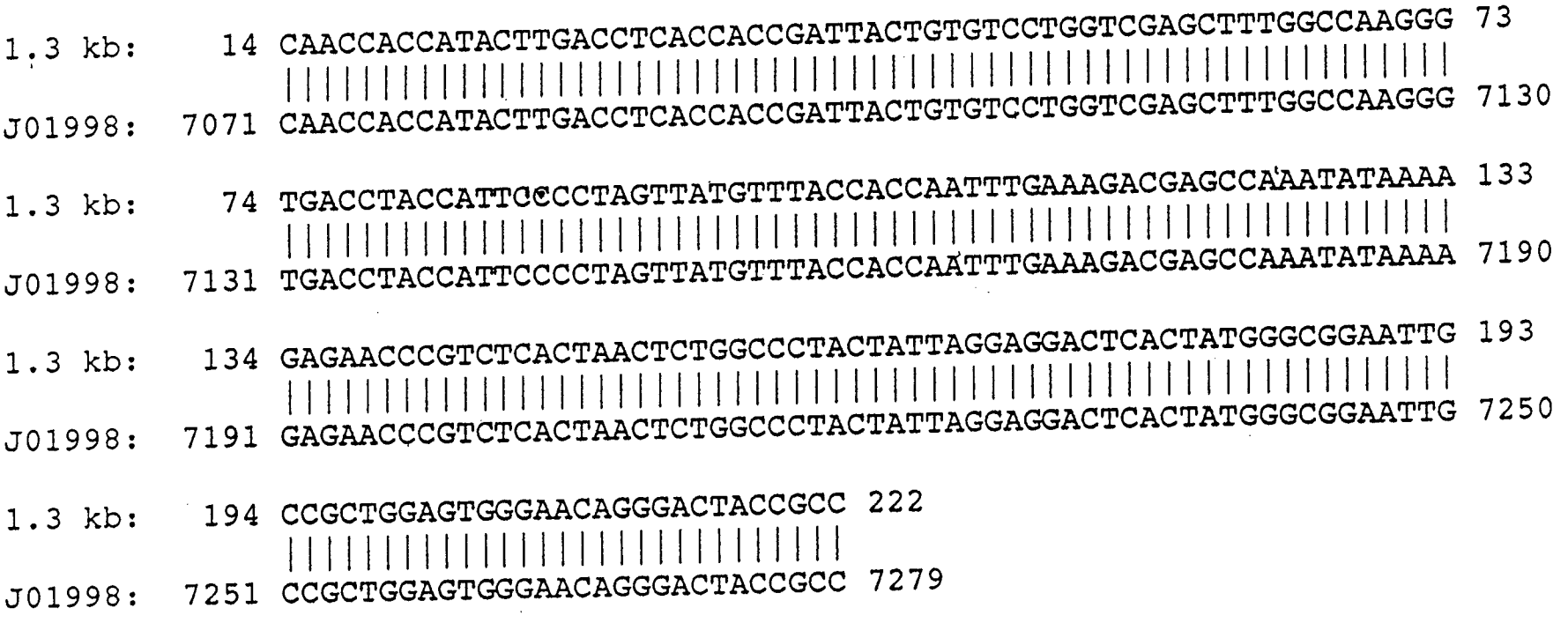
300000
200000
100000
0
MC38
MC38-CEA-2
Target Cells
Normal Mouse Sera
Group I Immune Sera
Group II Immune Sera
Group III Immune Sera
Anti-CEA MAb

Figure 2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1.3 kb: 134 GAGAACCCGTCTCACTAACTCTGGCCCTACTATTAGGAGGACTCACTATGGGCGGAATTG 193
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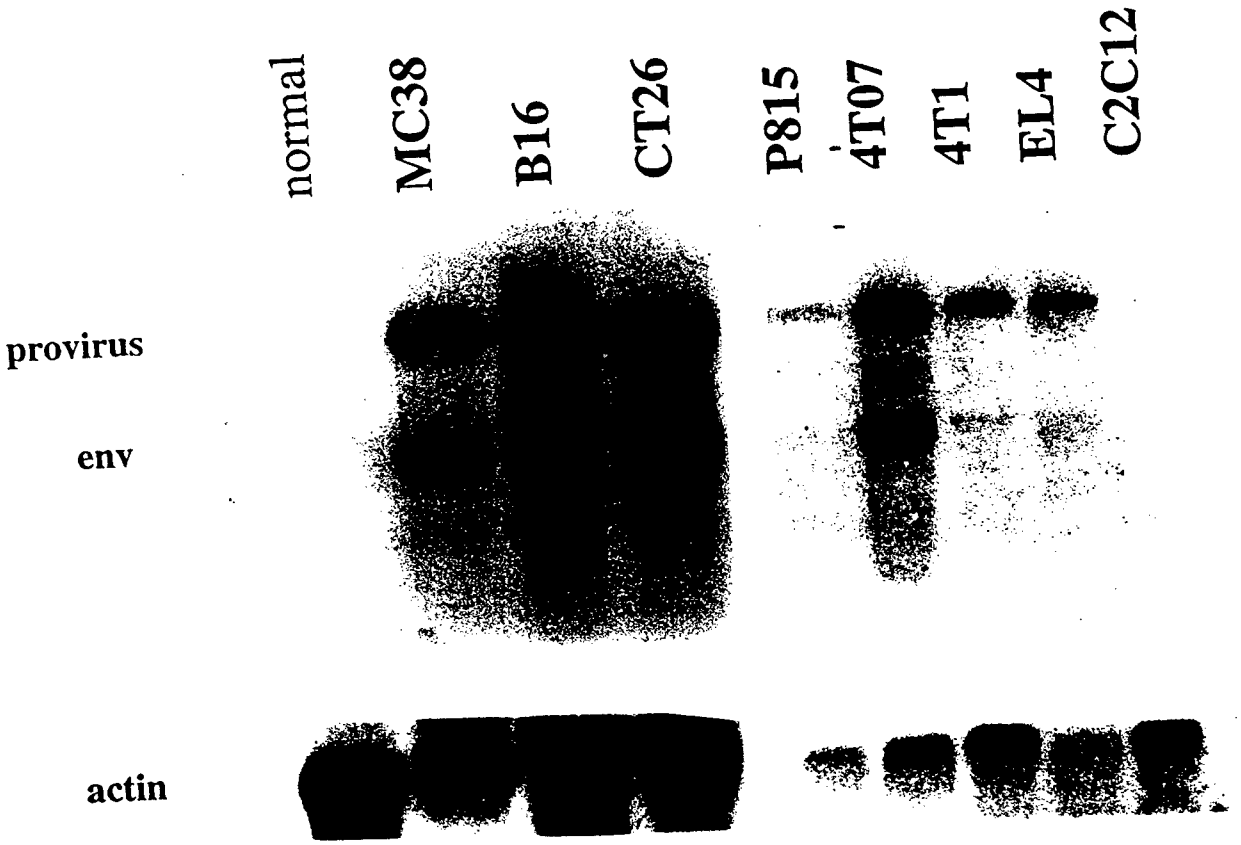
Figure 3A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CT26
P815
4T07
4T1
EL4
C2C12
provirus
env
actin

Figure 3B

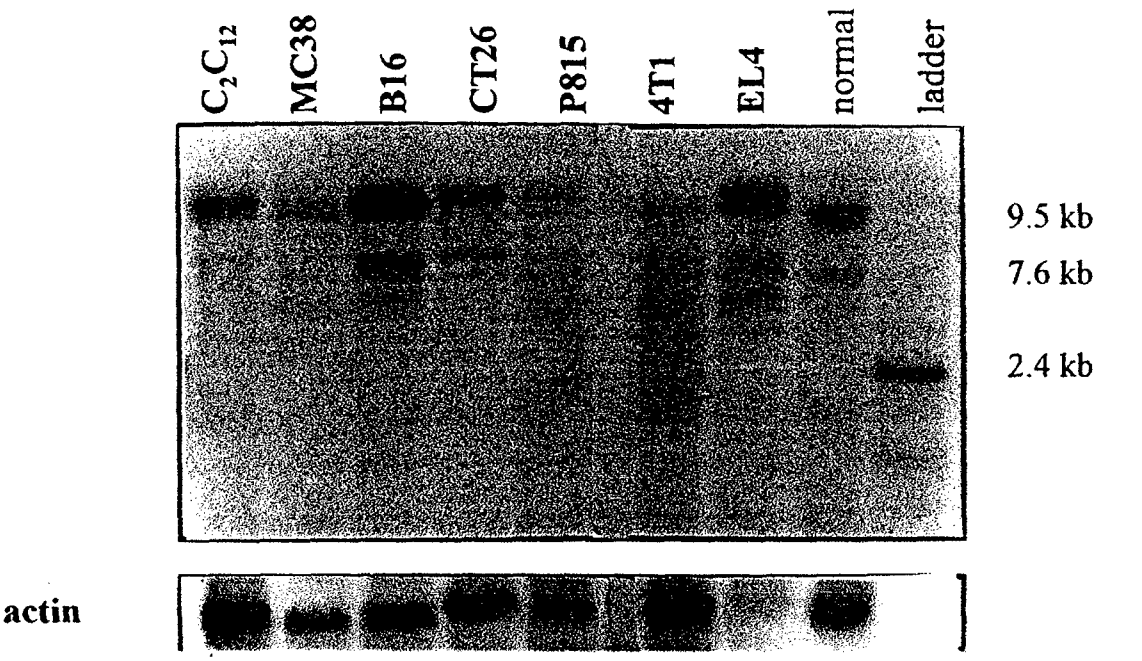
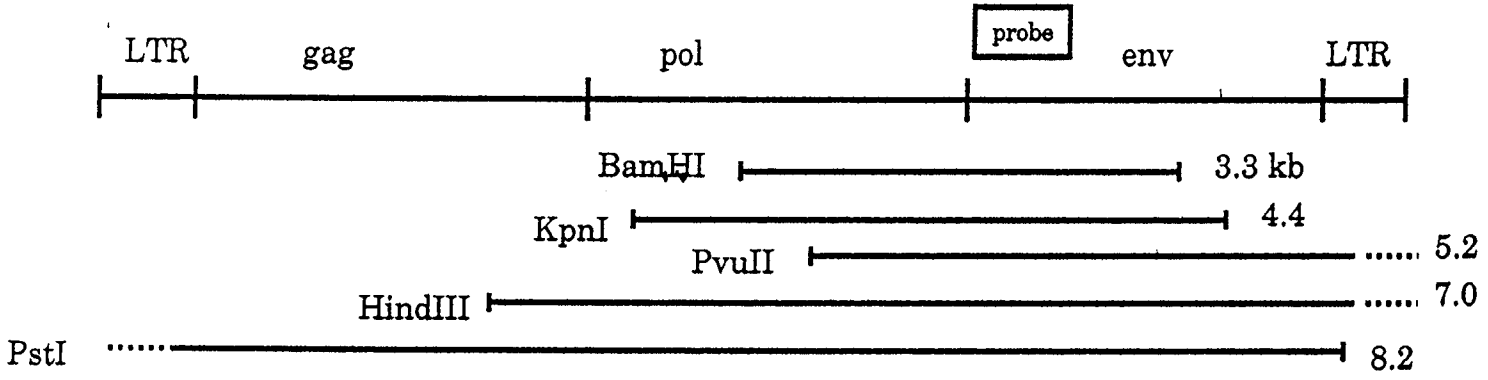

$C_2C_{12}$
MC38
B16
CT26
P815
4T1
EL4
normal
ladder
9.5 kb
7.6 kb
2.4 kb
actin

Figure 4A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HindIII
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PstI
8.2

Figure 4B

B

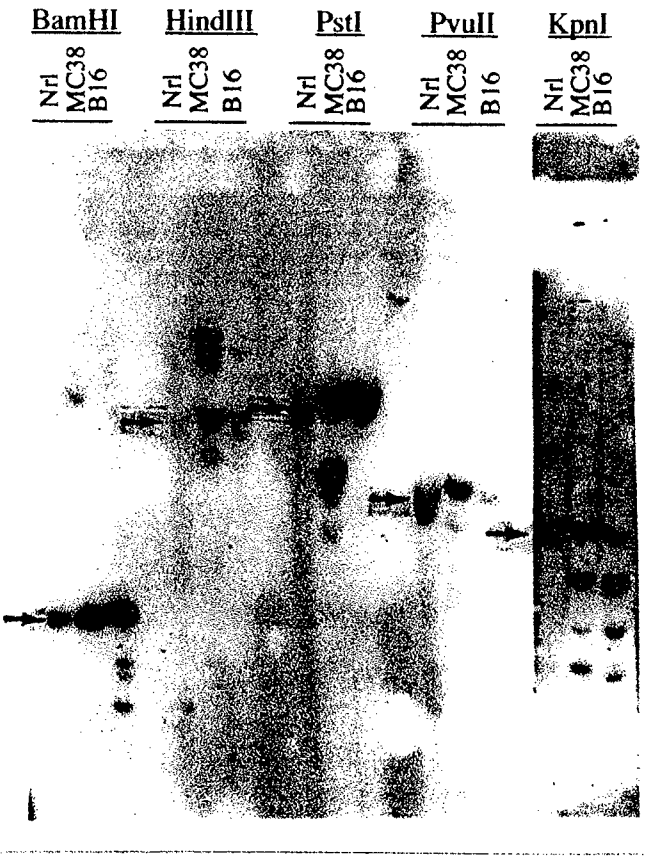
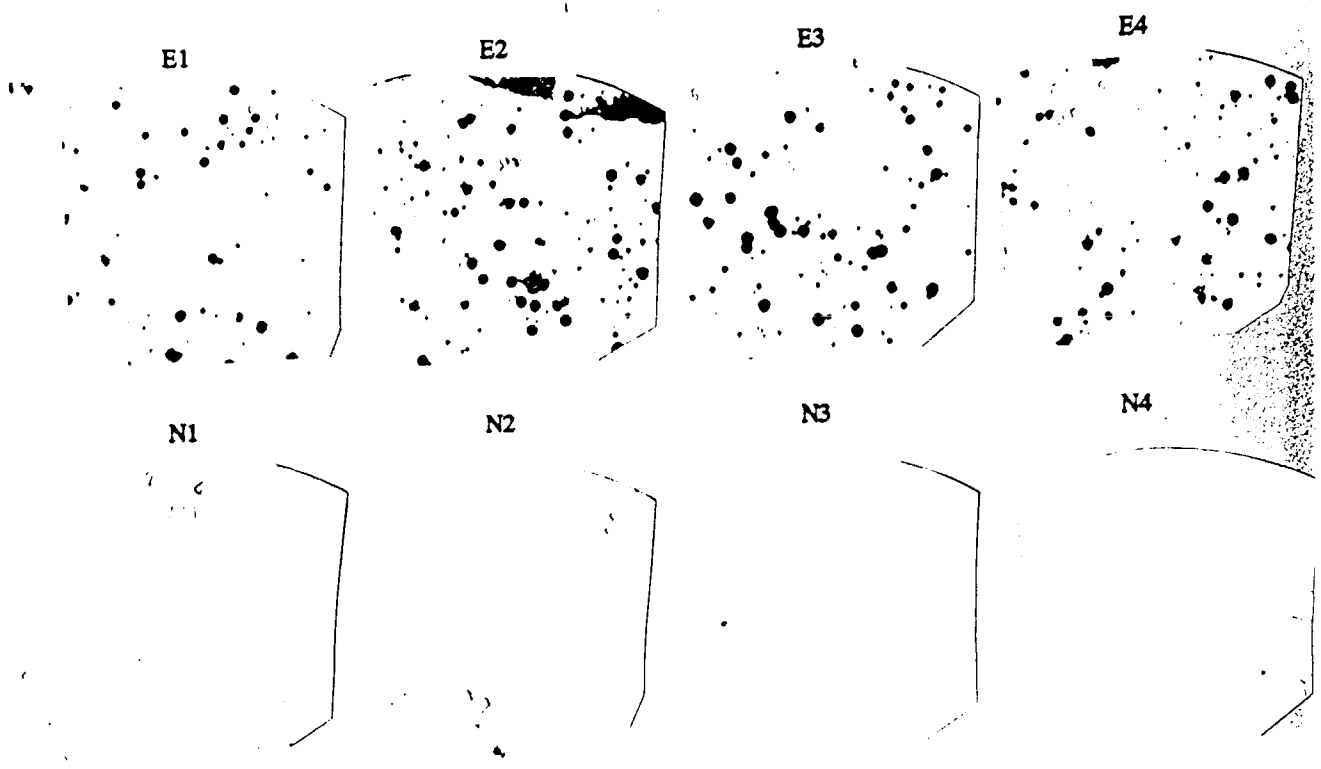


Figure 5



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